DORSALIZATION OF THE VENTRAL MARGINAL ZONE OF THE TRITURUS GASTRULA. I. AMMONIA-TREATMENT OF THE MEDIO-VENTRAL MARGINAL ZONE[1]

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Introduction

In the early gastrula of amphibians the material for the notochord and mesoderm is localized between the animal and vegetative hemispheres in the form of a ring. This marginal zone is most extensive at its medio-dorsal section, which extends well above the equator, and represents the broadest section of the ring. As one proceeds from this part towards the opposite medio-ventral section, the ring becomes gradually narrower, its upper boundary gradually lower, and its narrowest and lowest limit, the medio-ventral section, lies opposite the future dorsal lip of the blastopore.

The distribution of the presumptive organ rudiments of the trunk mesoderm within this zone reflects the dorso-ventral sequence of the rudiments in the completed embryo. Medio-dorsally lies the presumptive notochord, on both sides of it the presumptive myotomes, which are followed by the presumptive pronephros and lateral plate, and finally the presumptive blood island, which occupies the medio-ventral section of the marginal zone.

This sequence of the prospective values within the marginal zone is again reflected in the mode of differentiation of the isolated sections of the marginal zone cultured in Holtfreter solution (Holtfreter, 1938). Although here the differentiation of the individual sections does not faithfully realize their prospective value, the dorso-ventral sequence of morphogenesis is in its general form clearly recognizable. The ventral section of the marginal zone differentiates blood island, nephric tubules and the derivatives of the lateral plate. However, neither notochord nor muscle is formed in the isolate from this section. In contrast to this the differentiation of the notochord and muscle is the predominant feature of the isolated dorsal section. It must be kept in mind, however, that especially in the dorsal section the "regulative" tendency is at work to develop more than the prospective value, so that a part of the explant from the dorsal section has always the chance to become one of the more ventral mesodermal rudiments or even ectodermal tissue.

If comparable sections of the trunk mesoderm are isolated later (at the beginning phase of neurulation) and cultured in epidermis vesicles, the individual sections can be distinguished more clearly through the mode of their differentiation (Yamada, 1939, 1940). But even in this case the prospective value is not always

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faithfully followed. Further, it is demonstrated that by adding a piece of the presumptive notochord to the individual sections of the mesoderm of the neurula it is possible to alter the mode of differentiation of each isolate. This effect of the notochord consists in shifting the mode of differentiation of the isolate toward the dorsal side. The presumptive blood island which differentiates blood island, mesothelium and blood vessels in isolation, gives rise to nephric tubules and only a small quantity of blood cells, if it is combined with notochord material. The presumptive pronephros, which develops, in isolation, nephric tubules and blood cells, shows clear differentiation of muscle cells in the direct vicinity of the implanted notochord tissue. Presumptive myotome, which in isolation gives rise to nephric tubules besides undifferentiated myoblasts, develops muscle cells with definite fibrils on implantation of the notochord material. Thus the effect of the notochord on the morphogenesis of the mesoderm can be designated as "dorsalization." From these results it may be expected that also the isolated ventral marginal zone of the gastrula could be dorsalized by appropriate methods, perhaps with more profound results, since the material must be more plastic in the gastrula than in the neurula.

It is well known that the presumptive ectoderm of the early gastrula reacts to many chemical stimuli by undergoing neural differentiation. The most typical case may be the neuralization of the isolated gastrula ectoderm caused by a sublethal cytolysis, as demonstrated by Holtfreter (1945, 1947). Through brief treatment of the isolated gastrula ectoderm with a solution of extreme pH (higher than 9.4 or lower than 5.0), Ca-free Holtfreter solution, distilled water and 10 per cent alcohol, he obtained differentiation of brain, eye, placodes, mesenchyme and melanophores. Thus the cephalic ectodermal structures and mesectodermal components were induced, while the more caudal ectodermal structures, such as the otocyst and spinal cord, and the mesodermal structures were wholly absent. The arrangement and form of the induced rudiments were, however, highly atypical. The same seems to hold also for "self-induced" explants obtained by Barth (1942) and Holtfreter (1944) and for the explants of Shen (1942) cultured in a dilute solution of a hydrocarbon. Morphogenetic response of the isolated ectoderm to implantation of a protein belongs also to this category (Yamada, 1947). Here the induced structures are more diversified and typically organized than in the above cases.

All these inductions of the neural differentiation in the ectoderm are dorsalizations as defined above. This may become clear if one recalls the fact that epidermal differentiation is a ventral mode of differentiation, while brain differentiation is a dorsal one, according to the topographical relation which can be visualized, for instance, in a cross section of the anterior part of an early neurula. Between two extreme levels lie the intermediate levels of the sense organs, placodes and mesectoderm. Together they constitute the dorso-ventral scale of the anterior ectoderm. Induction or neuralization is then nothing but the conversion of the ventral mode into a more dorsal one. Thus the sublethal cytolysis dorsalizes the isolated ectoderm, which untreated tends to adopt the ventral mode of differentiation. Now in the double-potential theory proposed by the present author (Yamada, 1947, see below) the dorso-ventrality of the morphogenesis has a common basis in the ectoderm as well as in the mesoderm, and consequently the internal factor which regulates it in normal development may be of the same quality throughout the embryo. From this it may be expected that any factor which has a dorsalizing effect on the competent ectoderm can dorsalize the development of the mesoderm,

if it is given in an appropriate condition. This theoretical expectation is tested in the following experiments.

As the dorsalizing procedure a brief exposure to a solution of ammonia was adopted. At first this treatment was tested on the isolated ectoderm of the gastrula of *Triturus pyrrhogaster* (control series I), and after its dorsalizing effect was ascertained, the isolated medio-ventral marginal zone of the gastrula of the same species was treated in the same way (experimental series). Parallel to this series, the untreated medio-ventral and latero-ventral marginal zones were isolated and cultured as the control of the reacting system (control series II).

Ammonia was used in the experiment since it actually is concentrated in the dorsal regions of the amphibian embryo during the phase of gastrulation and neurulation (Boell, Needham and Rogers, 1939), and may have the role of a mediating factor in morphogenetic induction.

Material and Technique

The eggs of *Triturus pyrrhogaster* were obtained through implantation of ox hypophysis in the mature female, and fertilized naturally or artificially. Operations and culture were done under sterile conditions. The egg capsule was washed with 60 per cent alcohol for 50 seconds, thoroughly rinsed in sterile Holtfreter solution and then removed with fine scissors sterilized with dry heat. After freeing from the vitelline membrane with forceps, the embryos were operated on with a glass needle on the Schotté ring (Yamada, 1937). Standard Holtfreter's solution (pH 7.2–7.6) was used exclusively for operation and culture. Sodium bicarbonate for the Holtfreter solution was sterilized with 60 per cent alcohol and after drying dissolved in sterile distilled water and then added to the unbuffered solution which had been boiled and cooled beforehand. The solution of ammonia used for the shock treatment was prepared immediately before use by adding 0.01 cc. of 20 per cent ammonia solution to 10 cc. of Holtfreter solution. Its pH was roughly estimated to be 11.8–12.2. The condition of the laboratory did not permit the regulation of culture temperatures which fluctuated between 12° and 26° C. However, no essential effect was noted other than a variation in the velocity of development. The isolates were cultured in covered glass dishes of 25 cc. volume. After culturing for seven to eighteen days the isolates were fixed in Bouin's fluid, stained in bulk with borax carmine, sectioned at 12 μ and stained with picro—"Blauschwarz."

The developmental stage of the operated embryo was classified according to the following criteria: Stage I: The youngest gastrula with the first sign of the blastopore as a pigmented patch; Stage II: Gastrula shortly after formation of groove of invagination; Stage III: The blastopore crescent-shaped.

Control Series I: Shock Treatment of the Isolated Gastrula Ectoderm with Ammonia

It was expected that the isolated ectoderm of the early gastrula of *Triturus pyrrhogaster* would differentiate cephalic ectodermal structures if exposed temporarily to a solution of ammonia, just like the isolated ectoderm of *Triturus torosus* (Holtfreter, 1945, 1947).

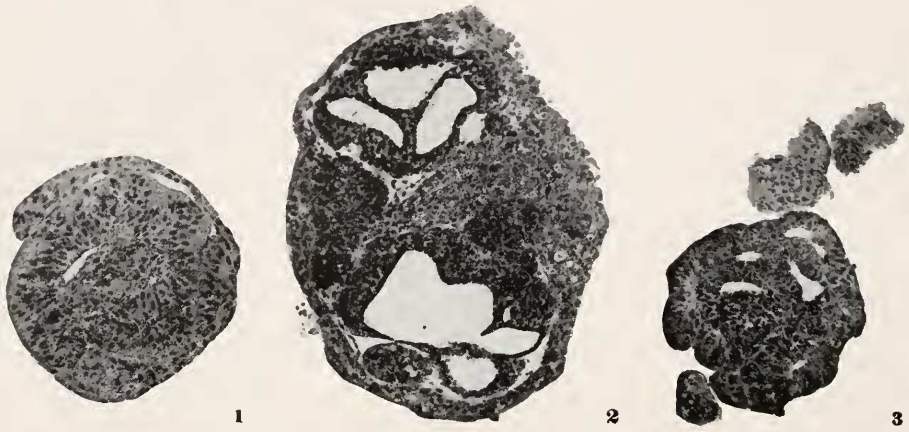
The presumptive ectoderm of the early gastrula was excised and put in the dilute solution of ammonia (see above). Special caution was taken not to include

in the isolate any cells from the marginal zone. After a latent period of ca. 50 seconds the explant began to disintegrate. The first sign was disruption and break-down of the pigmented surface into small fragments. This process was often accompanied by inverse curling of the explant edge, which resulted in the formation of a cup with the original internal surface occupying the convex outside. This cup persisted only for a while. Then the individual cells rounded up, became transparent and swollen and burst out of the aggregate. As the disaggregation progressed further a sticky transparent colloid was released. Thus within three to four minutes the original contour of the explants was almost lost. The velocity of the disintegration varied considerably, perhaps owing to the differences in the state of the curling and size of the explants. Before the explants were completely disaggregated they were transferred to another dish containing Holtfreter solution. Here the cells became less and less transparent and a part of them adhered to each other to form a solid aggregate of varying size, while other cells remained free on the bottom of the dish and were later discarded. Five explants were treated simultaneously with ammonia solution, and the number of the resulting aggregates varied from two to three. An aggregation of cells from two different isolates to form a common aggregate was a usual occurrence. The solid aggregate was then further transferred to the second dish with Holtfreter solution and cultured as usual. The surface of the aggregate was almost perfectly free of pigment and seemed to be occupied by the originally internal surface of the ectoderm. In the course of the subsequent three days a considerable number of cells were discharged from the reaggregated explant. In most cases the latter became a more or less regular, compact spheroid. No extensive adhesion to the glass bottom was observed. The formation of irregular small folds which are characteristic for the untreated ectoderm explant of this species occurred only in four of forty explants examined. On the other hand, no neural plate could be observed on the surface of our aggregates. This deserves attention in so far as the isolated ectoderm of this species shows a very clear neural plate under the inductive influence of chemicals and the organizer. However, by dissecting the aggregates two to three days after the ammonia-treatment, small strongly pigmented areas were found in its interior, scattered among the pigment-free cells. These areas are derived perhaps from the original surface of the ectoderm and have the significance of a neural plate. Probably neural differentiation originates in connection with them.

Of forty microscopically examined explants, thirty-one (77 per cent) showed neural differentiation. Four of the nine without neural differentiation belonged to the afore-mentioned type with many irregular folds. In sections, they cannot be distinguished from the untreated explant of *Triturus* ectoderm, being an unorganized mass of epidermis cells. The five remaining explants without neural differentiation are spherical in shape and consist of tightly condensed epidermal cells. Within them are encountered many cysts which often enclose cytolyzed cell masses besides densely pigmented cells, suggesting severe disturbance of the metabolism.

The structures encountered in the neuralized explants may be classified as brain-type, eye-type, neural mass, nose-type, lentoid, atypical neural balls, mesenchyme, chromatophores and frontal glands. It must be added, however, that the classification is of a rather provisional character and does not mean necessarily the definitive identity of the induced structure with the corresponding normal rudiments (see below).

Brain-type. Of twenty-nine brain-like bodies three show a rather normal form (Fig. 1). The typical appearance is often emphasized by the presence of nasal placodes closely applied to the "brain." In the other brain-like bodies the morphology is atypical in different degrees. The most atypical state is attained when an enormous body is furnished with a number of highly irregular lumina. One of six such cases is shown in Figure 2.



FIGURES 1–3. Neural tissues induced in the isolated ectoderm through exposure to ammonia solution. Fig. 1. A brain-like structure with a "nasal placode" on the right side. Fig. 2. A large explant showing two extensive brain-like structures with many lumina, accompanied by some placodes and mesenchyme. Fig. 3. An irregular brain-like vesicle accompanied by epidermal balls with frontal glands. × ca. 60.

Eye-type. In only two cases eye-like tissue is developed in connection with the brain-type differentiation. One of them is provided with a pigment layer, while the other is accompanied by a lentoid.

Neural mass. A group of neuralized cells without definite morphological characterization. This occurs regularly when small explants are perfectly neuralized and no epidermis exists to cover the neural tissue. Seven such cases are recorded.

Neural balls. This category also has no corresponding normal structure. It is probably heterogeneous, comprising some neural vesicles which are too small to be called a brain, and some bodies of placodal nature often enclosing large pigment-vacuoles. Eleven neural balls are differentiated in eight explants.

Nose-type. Of eighteen nasal placodes obtained, thirteen are closely applied to the brain and show a fairly normal condition (Fig. 1). Two others are differentiated without accompanying neural tissue.

Mesenchyme and melanophores. These elements are differentiated definitely only in one explant in close relation to the induced neural tissue. They cannot be distinguished from the normal ones.

Frontal glands. Typically differentiated glands are found in nine explants. They often form irregular groups with epidermal cells and are attached to the neuralized cell-mass as a protuberance (Fig. 3).

The size of the explants varied from 0.32×0.27 mm. to 1.28×1.05 mm. (expressed in the major and minor axes of the fixed explants, which are assumed

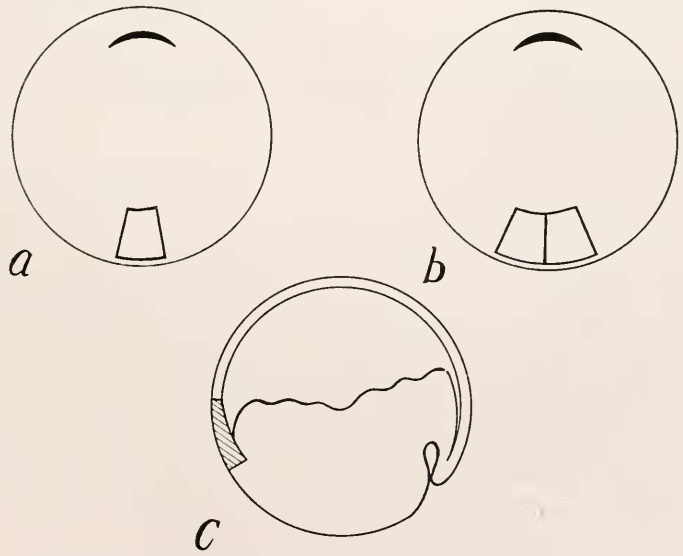
to be an ellipsoid). Neuralization occurred in the smallest as well as in the largest ones. Often the smaller explants are perfectly neuralized, while in the larger ones the morphogenesis of the induced structures progressed better. In six cases all the cells of the aggregate are neuralized so that the neural tissues are exposed on all sides to the medium. But even in such a state some lumina developed inside the neural mass and endowed it with a brain-like appearance (Fig. 3).

Summarizing, we can say that through the ammonia-treatment of the gastrula ectoderm only cephalic ectodermal or mesectodermal differentiations are induced, while the mesodermal or relatively caudal ectodermal differentiations are wholly absent. The results conform well with the findings of Holtfreter (1944a, 1947).

It may be added that the same treatment was carried out on a number of isolated pieces of ectoderm from the late gastrula and early neurula. They were all underlain with endomesoderm. Most of them did not recover from the disintegration. Only two explants from the presumptive epidermis of the gastrula with large yolk-plug were successfully cultured. Upon examination in sections, one of them shows the brain-type and the other the nose-type differentiation.

Control Series II: Differentiation of the Isolated Ventral Marginal Zone

A part of the ventral marginal zone from the early gastrula was isolated and cultured in Holtfreter solution. Two different ways were adopted to obtain the isolate. In series IIa two symmetrical sectors of the marginal zone were extended ca. 20° from the ventral median line on both sides (latero-ventral section), while in series IIb the sector of 20° lying exactly medio-ventrally was isolated (Fig. 4a–c). Utmost caution was taken against inclusion of the cells from the adjacent



Figures 4a–c. Scheme of operation. The early gastrula, in *a* and *b* viewed from the vegetative pole, in *c* shown in the median section. Fig. 4a. Operation for the "medio-ventral" section of the marginal zone. Fig. 4b. Operation for the "latero-ventral" section of the marginal zone.

sectors of the marginal zone in the isolate. The isolated pieces contained however, besides the cells of the marginal zone, some cells from the adjoining ectoderm and endoderm. This inclusion was intentional because for a good histological differentiation of mesoderm cells the presence of cells from other germ layers, which later furnish a covering tissue, is a necessary condition. The isolated piece was in most cases fused with other pieces into a single explant and cultured. The number of pieces constituting one explant varied from one to six. Several examples will be described:

VRK 108. From five gastrulae of stage II the medio-ventral sectors of the marginal zone were isolated and all were fused together in an explant and cultured in Holtfreter solution. One day after the operation the endo-mesodermal part of the explant formed a large whitish hemisphere, on which five ectodermal parts were attached separately as small cones. On the following days the ectodermal com-

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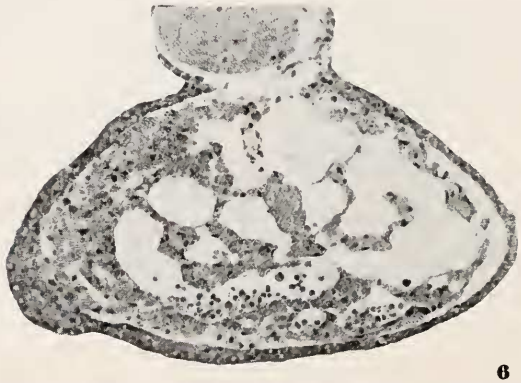

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FIGURES 5 AND 6. Development of the isolated ventral marginal zone. Fig. 5. Differentiation of blood island (in the center), enclosed in the mesothelium, externally covered by the epidermis. Fig. 6. Blood cells, blood vessels, and mesenchyme besides epidermal covering and undifferentiated endoderm. × ca. 60.

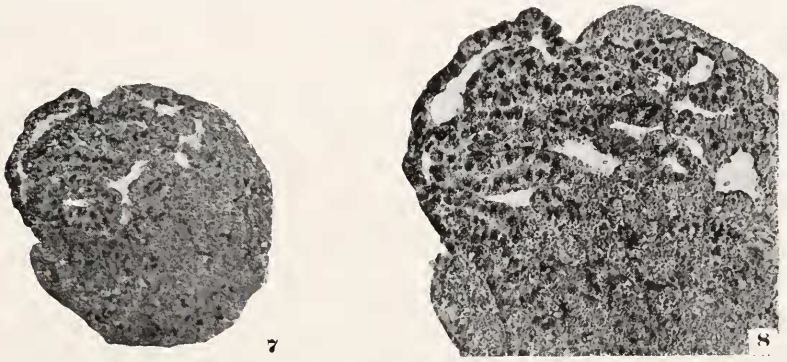
ponents gradually covered the surface of the endo-mesodermal hemisphere, and four of them fused with each other without losing their original individuality. Twelve days after the operation (culture temperature 21°–26° C.), when the large combined explant was fixed, it was found attached to the glass bottom with a cylinder of naked endoderm from which protruded a somewhat irregular, elongated body. It was covered completely with the epidermis, half-transparent and expanded. The size of the explant: 1.66 mm. × 1.28 mm.

Microscopical examination reveals that the protruded part of the explant is filled with loose mesodermal tissues: the yolk-rich mesenchyme, blood vessels enclosing blood cells and the mesothelium lining a wide space which is filled with blood cells (Fig. 6). Within the meshwork of mesenchyme and blood vessels is differentiated a piece of pronephric tubule, with one end attached to the endodermal cylinder.

VRK 5. From two gastrulae of stage II the medio-ventral sectors were cut out, fused together and cultured in Holtfreter solution. In the course of two days the explant formed a smaller compact sphere attached loosely to the bottom of the

culture dish. Cultured for fifteen days at 18°–23° C., the size of the explant: 0.84 × 0.78 mm.

Microscopical study shows that ca. three-quarters of the external surface of the explant is covered by endodermal epithelium. The latter is replaced by a well-differentiated mesodermal epithelium on the remaining surface. Within the mesodermal epithelium a cavity is formed which encloses a large amount of highly entangled pronephric tubules (Fig. 7). The tubules are connected with the external epithelium at some points. There exists no clear boundary between the tubules and epithelium and they both show a similar histological construction (Fig. 8).



FIGURES 7 AND 8. Differentiation of nephric tubules out of the isolated ventral marginal zone. A quantity of yolk-rich endoderm, no ectodermal component. Fig. 7, × ca. 60; Fig. 8, × ca. 100.

Obviously the external epithelium is nothing but the pronephric tissue modified by the topographical relation. Some sections of the tubules are free of yolk and better differentiated, while the others are laden with much yolk and seem to be retarded in differentiation. In all sections the central canal is poorly developed. A small quantity of mesenchyme, blood cells and melanophores are differentiated among the tubules. Embedded in the endoderm are some concentrated groups of mesoderm cells without any clear differentiation.

VRK a'. Operated as in the last example and cultured for eleven days at 19°–22° C. The explant developed into a spherical vesicle covered by the epidermis. Later two protuberances were formed at each pole, one elongated, the other spheroidal in shape. The size of the explant: 1.24 × 0.73 mm.

Microscopical study reveals that the main part of the highly expanded explant is filled with a large amount of blood cells enclosed in a cavity lined with mesothelium (Fig. 5). In the narrow space between the mesothelium and the epidermis are encountered a smaller quantity of blood cells, blood vessels, mesenchyme and a few melanophores. Each of the above-mentioned protuberances contains a part consisting of endodermal cells without any histogenesis.

The behavior of the explants as observed in the living condition may be summarized as follows. During the first several hours after isolation the explant acquired a more or less spherical form with expulsion of a small number of free cells. In the subsequent one to two days the pigmented ectodermal part of the explant became separated from the endo-mesodermal part by developing a promi-

nence which exhibited form changes of considerable degree. Thereafter the prominence retreated and the ectodermal part gradually fused to the endo-mesodermal ball, covering it more or less perfectly. In some cases the ectodermal prominence was constricted away from the endo-mesodermal part. Further morphogenesis of the explant is variable. It may develop a spheroidal compact body as in the case of the above described *VRK 5*, or a free spheroidal body covered by the epidermis as in the case of *VRK a'*, or in still other cases, a flat discoidal body firmly attached to the bottom and either covered or not covered by the epidermis. After the establishment of any of these forms further change in the external form was infrequent, except the enlargement through uptake of water from without, which often resulted in more or less transparent explants. In one case a minute tail-like formation developed from a discoidal explant (see below).

The results of the histological study of seventy-nine available cases are summarized in Table I. The explanted mesoderm has developed the following tissues: blood cells, pronephric tubules, mesothelium, blood vessels, mesenchyme and possi-

TABLE I

Differentiation of the isolated ventral marginal zone

Origin of explants	Number of explants	Frequency of tissues differentiated in the explants							
		Blood cells	Meso-thelium	Blood vessels	Mesen-chyme I	Mesen-chyme II	Melano-phores	Nephric tubules	"Somite"
Lateroventral (IIa)	44	34 77%	11 25%	9 20%	4 9%	3 6%	8 18%	25 56%	2 4%
Medioventral (IIb)	35	22 62%	6 17%	7 20%	7 20%	1 2%	1 2%	20 57%	1 2%
Total	79	56 70%	17 21%	16 20%	11 13%	4 5%	9 11%	45 56%	3 3%

bly melanophores. The table shows clearly that no significant difference can be recognized in the frequency of these tissues between series IIa and IIb (Table II). Therefore we can neglect for the moment this difference in operation and treat IIa and IIb together.

Blood cells. In most cases these occur in large numbers in a condensed group comparable to the normal blood island. In eighteen cases the blood cells are found in a voluminous cavity lined with mesothelium (Fig. 5). In twenty-four cases they are differentiated in a restricted space developed within the endoderm, or between the endoderm and the bottom of the culture dish. In fourteen other cases blood cells are gathered in a smaller group between mesenchyme or nephric tubules.

Nephric tubules. Tubules occur in the majority of cases in a restricted space between endoderm mass, or completely embedded in the latter, or (rarely) in the mesh-work of the mesenchyme and blood vessels. When they are perfectly embedded in the endoderm their histogenesis is very poor, but when they are bathed in the fluid of a cavity and invested with mesenchyme and blood vessels their histogenesis is more typical.

TABLE II

Significance of the origin of explants (medio-ventral or latero-ventral) and the number of pieces fused together for the differentiation of explants from the marginal zone

Origin of explants	Number of pieces fused together	Number of explants	Principal differentiations		
			Blood cells	Nephric tubules	"Somite"
Lateroventral (IIa)	2	44	34 77%	25 56%	2 4%
Medioventral (IIb)	1–2	26	15 58%	15 58%	1 3%
	5–6	9	7 77%	5 55%	0
Total		79	56 70%	45 56%	3 3%

It is interesting to note that between the blood cells and tubules the following relation exists:

(a) explants with blood cells but without tubules.....34 cases (43%)
(b) explants with tubules but without blood cells.....23 cases (29%)
(c) explants with both of them.....22 cases (27%)

Thus, all the explants have developed either one or both structures. The blood cells and tubules can be considered, therefore, as the main differentiations of the explants.

Mesothelium. A thin epithelium, lining rather voluminous cavities and developed within the epidermis, is considered under this heading. It is accompanied in most cases by blood cells, and often by blood vessels. In sections through explants with such a cavity, a colloidal constituent is precipitated in the form of finer granules or reticulum, which is stained intensively with "Blauschwarz." Quite probably the cavity formation is the result of the release of colloid from the mesothelium cells or mesenchyme cells.

Mesenchyme. Under mesenchyme two defined groups are encountered. One is characterized by large, yolk-laden cytoplasm and a very coarse mesh-work which is stained only slightly with "Blauschwarz" ("mesenchyme I" in Table I). The other is characterized by the yolk-free cytoplasm and slender processes intensively stained with "Blauschwarz" ("mesenchyme II" in Table I). In one explant in which the limb-bud is developed, mesenchyme II occurs in large quantities. Type I is far more frequent than type II and is observed mostly in association with blood vessels and blood cells.

Blood vessel. Blood vessels are formed either within the mesh-work of mesenchyme (Fig. 6) or just beside the mesothelium. Apparently they do not form a perfectly closed system. Blood cells are not always found within the vessels.

Melanophores. Out of nine explants with melanophores three do not possess an ectodermal component. In these cases, at least, it is probable that the melanophores are derived from the marginal zone cells and not induced from the ectoderm as a mesectodermal component.

Myoblasts. In a few cases a small part of the explanted mesoderm gave rise to a group of elongated cells which have the features of a myoblast. But in not a single case are definitive muscle cells with myofibrils differentiated. More often, condensed mesodermal cells are found in connection with the pronephric tubules. They may be considered as a fragment of an early somite before the myotome is segregated from another part of it. However, the differentiation did not progress further and we cannot designate these cells even as myoblasts.

Tail-like process. The above-mentioned minute process which occurred only in one explant, reveals itself in section as a very small tail-like process filled with mesenchyme II. No axial structures are found in it. But proximal to the process three small groups of mesoderm cells are found. One of them is composed of mesenchyme cells which are in the process of breaking up into free cells (of type II). Two remaining groups are compact but have apparently the same fate as the first. In none of the three groups is a differentiation of myoblasts discernible. Taken as a whole, the structure may be considered an incomplete tail without axial tissue. In other parts of this explant the mesoderm has differentiated nephric tubules and a small amount of blood cells.

Summarizing the observations of this series, it may be said that both the isolated medio-ventral and latero-ventral sectors of the marginal zone differentiate in the main: blood islands, nephric tubules and derivatives of the lateral plate. But in a very restricted number of cases (3.7 per cent) and in restricted quantity, some features of somitic development are observed, in no case, however, leading to complete differentiation of a myotome. Not a single explant differentiated notochord or definite muscle cells.

Table II shows further that the difference in the number of the pieces fused together did not have an important effect on the outcome of the differentiation so far as the present investigation is concerned. But from these restricted results it is not permissible to draw any general conclusions as to the relation between the quantity of the material and the quality of differentiation.

Comparing the differentiation of the explant with its prospective value, the following may be pointed out. The differentiation of blood island, mesothelium, blood vessels and probably also of mesenchyme I corresponds to the prospective value of the isolated region. But it is rather questionable whether the nephric tubules are contained in the prospective value of this section of the marginal zone, as maintained earlier by Holtfreter (1938). The somitic development which occurs very seldom and incompletely is surely not in accord with the prospective value of the isolate. Thus we must conclude that the main feature of development of the explanted ventral marginal zone corresponds to its prospective value, although there is a recognizable tendency to differentiate structures which lie more dorsally in the dorso-ventral scale of trunk mesoderm differentiation than the prospective value of the material.

Experimental Series: Shock Treatment of the Isolated Ventral Marginal Zone with Ammonia

In this series only the medio-ventral sector of the marginal zone as defined above (Fig. 4) was used as the material. The isolate, prepared in the same way as in the last series, was treated with 0.02 per cent ammonia solution until the disinte-

gration of the isolate took place, then transferred to Holtfreter solution where a part of the cells reaggregated. In this case more time was required for the disintegration than in control series I. This is perhaps due to the presence of a thick layer of yolk cells within the isolate, which are apparently more resistant to the dispersive action of the medium and protect the superficial layer of mesoderm and ectoderm from the action. An attempt was made to stop the treatment at a state of disintegration, which was comparable to that attained in the control series I. This was realized, however, only approximately, as the variability in the velocity and detailed course of the disintegration process was unexpectedly high. The period of treatment, which was determined according to the morphological state of disintegration, varied between 3½ to 7 minutes. One aggregate was obtained from a single original isolate, or from a number of original isolates. In the latter case the original isolates were treated with ammonia and the disintegrating masses were fused into one mass with the aid of a glass needle and transferred to a dish containing Holtfreter solution. Without further manipulation the cells reaggregated into a single body. In this way, two to ten original isolates were fused into a single aggregate. However, it must be kept in mind that the quantity of the resulting explant is much smaller than the sum of the original isolates, as a severe loss of cells occurs during and after treatment.

The morphological aspect of the course of disintegration of the marginal zone in ammonia solution, differed from that of the ectoderm in that the curling was insignificant and the process of actual disintegration was foreshadowed by a slow but steady rise in transparency of the cells. Some examples follow:

AVR 92. From four gastrulae of stage I, the medio-ventral piece of the marginal zone was isolated and treated with 0.02 per cent ammonia solution for ca. 4 minutes until the original contour of the individual isolates was almost lost, then transferred to Holtfreter solution. A severe loss of cells was observed at the time of treatment and during subsequent days, resulting in reduction of the size of aggregates. Large white endoderm cells, light brown mesoderm cells and dark pigmented ectoderm cells were discharged. Five days after the treatment the ovoid aggregate showed a small dark groove on one of its poles, while the rest of the surface was covered by the opaque, white endoderm. The explant adhered loosely to the bottom of the culture dish. Cultured for fifteen days at a temperature of 14°–24° C., the size of the explant: 1.81 × 0.90 mm.

Microscopic observations: Covered by an undifferentiated endoderm layer, a large complex of notochord and muscle developed (Figs. 9, 10). In one-half of the explant the notochord is only slightly curved and approximates the normal notochord of the corresponding stage in the diameter (0.15 mm.), while in the other half the notochord takes an irregular complex course and shows varying cross sections. Most of the notochord is composed of perfectly vacuolated cells and provided with a layer of connective tissue cells. The muscle tissue which accompanies the notochord is regularly segmented where the course of the notochord is more or less straight, but irregularly arranged where the course of the latter is complicated. The muscle tissue is provided with clearly differentiated myofibrils and a small amount of yolk granules, and invested with a layer of connective tissue cells, which seem to be derived from the original somite. Some elongated neural bodies are formed among the rather irregular complex of notochord and muscle. In some of its parts the neural tissue is laden with exceptionally abundant yolk granules,

and does not have a definite boundary against the neighboring mesoderm. It is difficult to identify the neural structure as any part of the normal nervous system, but as it is stretched in a slender form one can perhaps ascribe to it a caudal nature. A small group of the pronephric tubules are formed between muscle, notochord and endoderm. A mesodermal protuberance exposed to the medium is formed. This looks like a limb-bud, but is partly continuous with a fragment of neural tissue.

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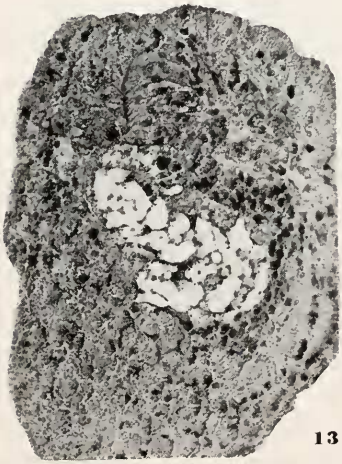
FIGURES 9 AND 10. Differentiation of notochord, muscle and neural tissue from the isolated ventral marginal zone after exposure to ammonia solution. Two sections through the same explant. Fig. 9. Notochord (right) and muscle (left) enclosed in the endoderm. Fig. 10. Somewhat irregular notochord accompanied by the muscle (left) and neural tissue (below). × ca. 60.

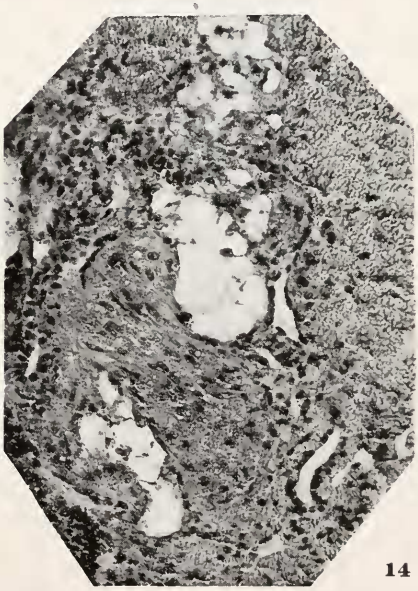
AVR 152. Three pieces of the medio-ventral section of the marginal zone were isolated from gastrulae of stages I and II and treated with ammonia solution for ca. 3½ minutes. Before the disintegration set in they were put back into Holtfreter solution. The aggregate formed a somewhat deformed sphere with small pigmented cell-groups protruding from its otherwise smooth whitish surface. An appreciable quantity of cells were discharged. Cultured eight days at 22°–24° C., the size of explant: 1.40 × 0.65 mm.

Sections through the explant show that the center of the ovoid explant is occupied by a piece of notochord with very irregular contour (Fig. 13). The maximal diameter is found to be 0.17 mm., a value which exceeds the value of a normal notochord of the corresponding stage. Some of the notochord cells are in an earlier phase of the vacuolization showing numerous small vacuoles within the cytoplasm, while others are completely vacuolated, retaining a minimal amount of cytoplasm. The notochord is incompletely surrounded by myotomes. In the periphery of each myoblast, the myofibrils are differentiated and enclose internally a good quantity of yolk granules. In some parts of the explant both tissues, the notochord and myotome, are not perfectly delimited, having a transitional zone in which the cells show an intermediate character. To one end of the notochord is attached a piece of neural tissue which is devoid of a definite structure, and is laden with abundant yolk. Some of the myotomes are connected with this neural tissue

without clear boundary. The external covering of the explant is chiefly a layer of endoderm.

At the pointed end of the explant the covering is ruptured by the above mentioned neural tissue with one extremity of the notochord embedded in it. The myotomes are directly applied to the endodermal covering, except in the region where a group of nephric tubules are formed between them. No blood island or mesothelium is differentiated.





FIGURES 11–14. Different types of notochord induced in the ventral marginal zone through ammonia-treatment. Fig. 11. A straight cord composed of perfectly vacuolized cells, accompanied by myotomes on the left side. Fig. 12. A typical young notochord, in which the vacuolization has progressed farther toward one end (left in the picture) than toward the other. Fig. 13. Rather atypical differentiation of the notochord, not clearly delimited from the surrounding myotomes (above and below) and neural cells (right). Fig. 14. In the upper third of the figure notochord cells are scattered within a field of neuralized cells and myoblasts. In the lower two-thirds an entangled notochord is formed within the muscle tissue. At right, below nephric tubules. All figures × ca. 100.

AVR 32. Three pieces of the medio-ventral marginal zone were isolated from gastrulae of stage I and immersed in ammonia solution. The treatment was continued until the isolates began to disintegrate into separate cells (ca. 5 minutes). In Holtfreter solution, the cells aggregated into an elongated, compact group which, in the course of cultivation, adhered loosely to the bottom. A rather small number of cells were set free. When the aggregate was fixed in Bouin's fluid after thirteen days of culture at a very low temperature (12°–22° C.), it was covered by a whitish layer of endoderm, through which an elongated dark field was vaguely visible. The size of explant: 1.78 × 0.89 mm.

Microscopic observations: A large piece of notochord (length ca. 1.2 mm.) runs along the long axis of the explant. Consistent with the low temperature it shows the first sign of its histogenesis. One end of this piece, which may be called "caudal" because of its delayed histogenesis, is twisted in a spiral, with its highly flattened, yolk-laden cells arranged in a single row like a pile of coins. The cytoplasm is almost devoid of vacuoles. The middle part of the piece is almost straight (Fig. 12). The arrangement of flattened cells in a single row is very accentuated and regular. Small vacuoles are scattered at random throughout the cytoplasm. In the "cephalic" third of the notochord the vacuolization has progressed further. Vacuoles are enlarging, cells are consequently expanding and are not as flattened as in other parts (Fig. 11). The nucleus is polygonal in cross section, being enclosed in a larger vacuole. The diameter of the notochord equals that of the normal trunk notochord at the corresponding stage (0.11 mm.). The notochord is surrounded on all sides by the mesoderm, which shows at some points the first sign of myotome formation. Near each end of the notochord a small cavity is formed lined by a columnar epithelium with a pigmented surface. They seem to represent the neural tubes. Both tubes are at many points continuous with the adjoining mesoderm, which is developing into myotomes. Moreover, at the "caudal" end the notochord is directly continuous with the wall of the neural tube. An endoderm layer with an uneven surface covers all these structures.

Observations during the cultivation may be summarized as follows. A large number of cells were discharged just after the treatment and also during the first three days of cultivation. Not only smaller ectodermal and mesodermal cells, but also large yolk cells were found among the free discharged cells. After the recovery, the external surface of the aggregate was mostly covered by the whitish endoderm layer. The ectoderm, if present, withdrew into the interior, often leaving a small neuralized fragment on the surface, except in several cases where the epidermis covered one part of the explant. As a rule, the mesoderm occupied the interior of the explant. The form change during the subsequent cultivation was

TABLE III

Differentiation of the isolated medio-ventral marginal zone treated with ammonia

Number of explants	Frequency of rudiments and tissues differentiated in the explants									
	Noto-chord	Muscle	Myo-blasts	Limb-bud	Nephric tubules	Mesen-chyme II	Melano-phores	Blood cells	Neural tissue	Otic vesicle
40	30 75%	25 62%	16 40%	2 5%	29 72%	10 25%	8 20%	2 5%	30 75%	2 5%

pronounced only in three cases where the tail developed from the explant, covered partly by the epidermis. A much less pronounced protuberance often developed on the surface covered by the endoderm. The free spherical, half-transparent body covered by the epidermis, often observed in control series II, did not occur. The explants were cultured for eight to seventeen days at room temperature before fixation in Bouin's fluid.

The results of the microscopic examination are summarized in Table III. Notochords obtained here have a varied appearance. Of thirty cases with notochordal differentiation, eight show only a small number of scattered vacuolated cells among the myotomes, while in other cases (twenty-two explants, 55 per cent of all available cases) a more or less defined notochord is differentiated. Of these cases six show an almost typical notochord with the diameter corresponding to that of a normal one. Very often (sixteen cases) the notochord occurs in an irregularly

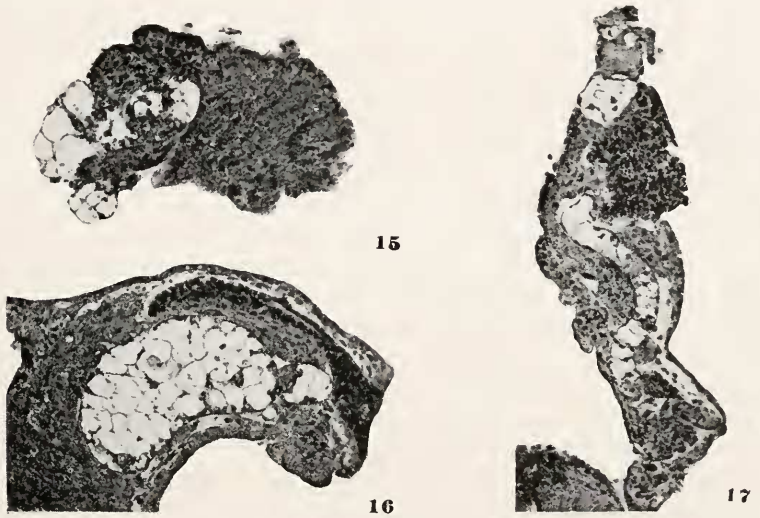


FIGURE 15. Uncovered notochord from the isolated ventral marginal zone treated with ammonia, accompanied by neural tissue and muscle cells.

FIGURE 16. A large notochord with myotome and spinal cord, induced with ammonia-treatment. The upper side of the explant is covered by the epidermis, the lower side with endoderm.

FIGURE 17. A tail-like structure developed from the explant, shown in Figure 16. A spiral notochord, with irregular mass of the myotome and neural tissue. All figures × ca. 60.

twisted form or in an aberrant form. These are found among neural tissue, myotomes or undifferentiated endoderm, exhibiting a great variation in the size of each vacuolated cell. Often cell types, which are intermediate between notochord, neural tissue and myotome, accompany these atypically formed notochords (Fig. 14). In only two cases the induced notochord developed a tail with accompanying neural tube and myotomes (Fig. 17); while in the other cases one might ascribe to the notochord the trunk character, since it possesses a larger diameter and does not show any tendency of pronounced stretching (Fig. 16). In most cases the notochord occurs within a covering tissue, but it may be exposed to the medium in the form of an unorganized tissue fragment (Fig. 15).

With a few minor exceptions the notochord is accompanied by myotomes and neural tissue. The myotomes attain a good differentiation and typical arrangement only when a typically formed notochord exists in their direct vicinity. The neural tissue and notochord have also an intimate relation. Often the fragments of notochord tissues are embedded in the neural tissue (Fig. 15) or are enclosed by a neural epithelium. Also, in the other cases, both tissues have the tendency to occur in association. Only two explants belonging to the group of imperfect notochordal differentiation are devoid of neural tissue. Here only a small number of notochord cells are scattered among the myotomes.

Neural tissue is formed very frequently (in thirty out of forty cases or 75 per cent). In twenty cases, it is differentiated on the surface of the explant without any characterization and cannot be identified with any part of the normal nervous system. The same holds for the aberrant neural tissues developed inside the explants (six cases). Only in three cases is a fragment of the spinal cord differentiated which runs along the notochord (Fig. 16). In two cases the neural cord is found as a component of the tail. Their morphogenesis is rather atypical. Differentiation of a brain cannot be ascertained in any explant. In two cases a structure developed which resembles the otic vesicle. One of these is accompanied by two ganglion-like cell groups. No nasal placode or lens is observed. Most of the neural tissues are partly or wholly laden with an exceptionally large amount of yolk granules. A remarkable consequence of this is the occurrence of clearly differentiated neurofibrils just beside the dense group of yolk granules. In younger explants the neural tissue and mesoderm have no clear limiting surface and one gets the impression that both tissues are derived from a common material.

A myotomal differentiation is found in thirty-nine of forty explants (97 per cent). Of them, twenty-five show the muscle cells with myofibrils, while other explants contain only myoblasts without fibrils. In at least five of the latter cases the explant was fixed too early for fibril differentiation to have occurred. The myofibrils exhibit the best differentiation when a normally formed notochord runs in the vicinity of the myotome. In these cases the parallelism between the course of the notochord and muscle cells is very conspicuous. Aberrant formation of the notochord is paralleled by disturbed arrangement of the muscle cells. There exists an almost perfect correlation between the occurrence of notochord and the differentiation of the myofibrils: (1) If the notochord is absent, fibrils are not differentiated (five cases); (2) In the presence of scattered notochordal cells, half of the explants differentiated fibrils (ten cases); (3) In the presence of a well defined notochord the fibrils are always differentiated (nineteen cases, excluding three prematurely fixed cases). A limb-bud is formed only in two explants. One of them shows an extensive proliferation of mesenchyme cells (type II). The nephric tubules are developed in twenty-nine out of forty cases. They are better differentiated when the explant contains notochord and muscle as well. A corresponding situation was found by the author in isolated mesoderm from the neurula. This influence of the axial organs on the development of the nephric system deserves further analysis. The blood island is found only in one case, in a restricted cavity between the endoderm and bottom of the culture dish. In no case did a cavity, lined with the mesothelium or filled with yolk-rich mesenchyme and blood vessels, develop in the explant.

Since in this series the number of the explants fused together varies considerably, it seems advisable to inquire whether the frequency of the notochord differentiation depends upon the number of fused pieces. In Table IV, the relation between the notochord differentiation and the number of original pieces is summarized. It appears that the notochord can develop in the explants from one isolate as well as in those from ten isolates. No significant difference in notochord frequency seems to exist between groups of one to three isolates.[2] It is probable

TABLE IV

Relation between the number of the original isolates fused together and the frequency of notochord differentiation

Number of isolates fused together	Number of cases	Explants with notochord differentiation	Frequency of notochord in %
1	14	10	71
2	11	8	72
3	12	9	75
4	1	1	(100)
6	1	1	(100)
10	1	1	(100)
Total	40	30	

that in explants with more than four isolates the frequency is higher, although the small number of cases does not allow any definite conclusion. On the other hand, the morphogenesis of the induced notochord may be influenced in some degree by the size of the explant. In explants from one isolate the notochord cells occur in small scattered groups without forming a cord-like tissue, while in explants from more than two isolates a more or less typically organized notochord can be developed.

Results of this series of experiments may be summarized as follows. The isolated, medio-ventral sector of the marginal zone of the earlier gastrula together with a small amount of adjacent ectoderm and endoderm, differentiates notochord, muscle and nephric tubules as well as neural tissues, if it is briefly exposed to an ammonia solution and then cultured in Holtfreter solution.

DISCUSSION OF RESULTS

An appropriate understanding of the results of control series I may be possible only when the behavior of the untreated explant of the ectoderm of a gastrula of *Triturus pyrrhogaster* in Holtfreter solution is known. Such explants differentiate into nothing but atypical epidermis. This result, confirmed repeatedly by the author and described in another paper in preparation, shows clearly that the isolated ectoderm of this species behaves like that of *Triturus torosus* and does not respond to isolation and cultivation by undergoing neural differentiation as does the isolated

[2] The significance of the difference in frequency of the notochord in the explants from one isolate and in the explants from three isolates in the above table, was tested after the exact treatment of R. A. Fisher and the probability of occurrence of the observed set of frequency was found to be 0.795. Hence we *cannot* conclude from the above data that notochord occurs in the explants from three isolates more frequently than in the explants from one isolate.

ectoderm of *Amblystoma punctatum*. Hence the neural differentiation obtained in control series I must be ascribed to the ammonia treatment. Study of the induced structures in this series has shown that they have more or less distinct characteristics of cephalic ectodermal rudiments and tissues such as brain, eye, nasal placode, lens, mesenchyme, melanophores and frontal gland. In another paper will be presented data which show that comparable differentiations occur in the isolated ectoderm induced by a multitude of substances and also by a living cephalic organizer; the similarities between these induced structures and corresponding normal structures cannot be a mere coincidence. The conclusions conform in general with those obtained by Holtfreter (1944a, 1945, 1947) in so far as the "self-induced" explants of *Amblystoma punctatum* and the imperfectly cytolyzed explants of *Triturus torosus* are concerned.

Comparing the results obtained in control series II and the experimental series it seems likely that the ammonia-treatment induces in the isolated medio-ventral marginal zone the differentiation of notochord and muscle, and suppresses the development of blood cells and mesothelium. But before this can be concluded we must exclude two possible sources of error: (1) *The development of dorsal tissues may be caused by the presence of some cells of the adjacent marginal zone taken along erroneously at the operation.* This possibility seems to be excluded, because in control series II it was shown that even the latero-ventral section never gave rise to notochord and muscle. It may be remembered that the dorsal limiting line of this section lies 10° more dorsal than that of the medio-ventral section, which was exclusively isolated in the experimental series (Fig. 4). (2) *The differentiation of the dorsal tissues may be brought about by the volume increase following the reaggregation of cells from many isolates into a single aggregate.* We have shown above that axial differentiation is possible even in the ammonia-treated explant originating from one isolate and having a smaller quantity than the average explant of control series II. On the other hand, in control series II, typical differentiation of the ventral mesoderm occurred even in the explants originating from six pieces of medio-ventral section. The second possible source of error can also be rejected.

Thus, it may be concluded that through ammonia-treatment the mode of differentiation of the isolated medio-ventral marginal zone can be altered, so that ventral mesodermal differentiations such as the blood island, blood vessels and mesothelium are suppressed, whereas dorsal differentiations such as the notochord and muscle are initiated, and intermediate differentiations such as the nephric tubules are not significantly altered (Table V).

The close parallelism and intimate spatial relation between the notochord and neural tissues observed in the experimental series deserves special attention. This correlation can be most readily explained if we assume that the neural tissue is induced in the ectodermal component of the explant by that part of the mesoderm which later differentiates into notochord and muscle. However, it must be recognized that the following possibilities are by no means excluded: (a) The neural differentiation and notochordal differentiation are induced separately in the ectodermal and mesodermal component respectively by the ammonia-treatment. The observed spatial relation between both tissues results from a later mutual approach and fusion. That such mutual approach occurs between tissues of the same nature is abundantly exemplified (Yamada, 1937; Holtfreter, 1944b) and it is not improbable that an analogous attraction also exists between different tissues of the

TABLE V

Comparison of the mode of differentiation of the ammonia-treated and non-treated isolate from the ventral marginal zone

Differentiation	Frequency of tissues in %	
	Control series II (non-treated)	Experimental series (ammonia-treated)
Notochord	0	75%
Muscle	0	62%
Mesenchyme II	5%	25%
Melanophores	11%	20%
Nephric tubules	56%	72%
Blood cells	70%	5%
Blood vessels	20%	0
Mesothelium	21%	0

axial region. (b) One part of the neural tissue obtained did not originate from the ectodermal component but from that part of the mesoderm which otherwise became notochord and muscle. Holtfreter (1938) showed that the dorsal marginal zone has the tendency to differentiate neural and other ectodermal tissues, besides notochord and mesodermal tissues, if it is isolated and cultured in Holtfreter solution. If, in the present case, ammonia-treatment makes the ventral marginal zone equivalent to the dorsal marginal zone, it is very probable that the same process can occur in these explants. Indeed, the morphogenesis of the ammonia-treated isolate of the ventral marginal zone does not differ much from that of the isolated organizer. As repeatedly pointed out above, a part of the neural tissue obtained in the explant shows intimate connections with the mesoderm and often a very high content of yolk granules. These facts can be most readily explained if we assume these possibilities: (c) One part of the obtained notochord did not originate from the mesodermal component but from the ectodermal component of the isolate, which was otherwise neuralized. This is not impossible since the presumptive ectoderm can be induced to form notochord and muscle, if it is applied to the trunk mesoderm of the neurula (Holtfreter, 1933). (d) The differentiation of the notochord was evoked in the mesoderm by the presence of the ectoderm which was neuralized by the treatment. This possibility might seem to contradict the observed fact that the neuralized tissue forms (in control series I) rudiments of more or less distinctive cephalic type, while in the experimental series the neuralized tissue shows characteristics of trunk and tail. This fact apparently supports the first assumption that the neural tissue is induced secondarily by the notochord and mesoderm. In spite of this we must not completely neglect the possibility that the neuralized ectoderm acts as an inducer on the mesoderm, while the subsequent differentiation of the ectoderm is inversely governed by the mesoderm or notochord and, consequently, the neural tissue acquires a trunk or tail nature (Yamada, 1939).

It is probable that many of these possibilities are realized in our experiments. An experimental test of these possibilities is not as simple as one might expect, as the boundary between the ectoderm and mesoderm is very indistinct on the surface of the early gastrula, and some observations suggest that the induced differentiation of the notochord is connected with this boundary. This problem will be the subject of a further series of experiments.

Theoretical Considerations

In order to discuss the theoretical significance of the present results, it seems necessary to introduce the double-potential theory proposed by the writer in an article written in Japanese (Yamada, 1947), as it furnishes the basis for the present investigation.

The essential points of the theory can be described most concisely as follows: (1) The developmental activity of a given germ region depends, above all, upon two sorts of "potentials," [3] which are designated respectively the dorso-ventral and cephalo-caudal, and can vary relatively independently of each other. (2) At a given developmental stage, the dorso-ventral and cephalo-caudal potentials show a definite gradient along the respective axis; or, otherwise expressed, every germ region has a definite value of both potentials according to its topographical position relative to both axes. (3) In the course of development the absolute values of both potentials show a continual change, which is characteristic for the germ region and for the stage. Thus the developmental activity of any germ region may be defined as the combined effects of the time-curves of both potentials. (4) The change in potential is effected partly through the factors inherent in the germ region and partly through factors external to it. The latter factors are called the mediators. There are two of them . . . the dorso-ventral and cephalo-caudal mediators affecting respectively the dorso-ventral and cephalo-caudal potentials.

This theoretical scheme is constructed to deal with the early ontogenesis of amphibians, but it is expected that the same principle can be adapted to other developmental systems, with alterations of unessential points.

To apply this scheme to the present problem, we have to follow some suggestions made earlier: (a) Considering the analysis of the normal course of development and the experimental data, it was assumed that the dorso-ventral potential is connected primarily with the intensity of the biochemical activity of the developing system, while the cephalo-caudal potential is connected primarily with the morphogenetic movements of the developing system. (b) Most of the phenomena, called embryonic induction, involve a shift of the dorso-ventral potential toward the dorsal end, and may be in this sense called dorsalization. The dorso-ventral mediator, which is responsible for this effect, seems to be (in most cases) chemical in nature. Many chemical substances or procedures can play the role of a dorso-ventral mediator. (c) The morphogenetic indifferent state, such as is found in the presumptive ectoderm of the early gastrula of many amphibians, is characterized by the ventral (low) value of the dorso-ventral potential and the cephalic (low) value of the cephalo-caudal potential. This means that through the action of any dorso-ventral mediator, such a germ region can be induced to form cephalic axial organs (eye, brain and nose), while without such action of mediators this germ region will differentiate only epidermis in the urodeles and epidermis and sucker (the cephalic and ventral differentiation) in the anurans. If we want to get caudal axial organs out of the same material, the cephalo-caudal mediator must be applied as well as the dorso-ventral mediator, to shift the cephalo-caudal potential in caudal direction. The ventral value

[3] The concept of "morphogenetic potential" was first introduced by Dalcq and Pasteels (1937) in their theory of morphogenesis. As will be clear from the following text, in our theory a meaning is given to this concept which differs much from that in the theory of the Belgian authors. However their basic contributions to the theoretical problem here treated must be appreciated.

of the dorso-ventral potential and the cephalic value of the cephalo-caudal potential being called "low," the effect of the dorso-ventral and cephalo-caudal mediator can be designated, in this case, as a "rise" in respective potential value. (d) If a germ region with a high value of one of the potentials comes in contact with a germ region with a low value of the same potential, the former generally acts as the mediator, provided that an appropriate contact surface is established. The organizer acts through the coordinated spatio-temporal pattern of both mediators, which is found in the potential composition of the germ region concerned, i.e., the dorsal marginal zone.

From the standpoint of the double-potential theory the morphogenetic effect of sublethal cytolysis can be designated as dorsalization; in other words it raises the dorso-ventral potential of the developing system. If the presumptive ectoderm of the early gastrula, with low values of both dorso-ventral potential (*Pdv*) and cephalo-caudal potential (*Pcc*), is treated with a sublethal dose of the cytolyzing agent, the *Pdv* will be raised to a higher value while the *Pcc* will be left unchanged. The end result must be a dorsal cephalic differentiation (such as brain and eye) without additional caudal differentiations (ear vesicle, spinal cord, notochord and muscle). The facts correspond well to this expectation. One of the most striking and, until now, unexplained features of the "self-induced" ectoderm is that it comprises almost exclusively structures of the foremost region of the head (Holtfreter, 1948) and is thus just what is to be expected from our theory.[4] The qualitative difference within the obtained cephalic inductions may be attributed, by this theory, to the difference of *Pdv*-curve and its spatial distribution in each case. A sharp rise of the *Pdv*-curve leading to the maximal value may be followed by differentiation of the eye. A less pronounced elevation may lead to the brain. A still less pronounced one corresponds to the nose-type, and a weak one, perhaps, to the mesenchyme and chromatophores. A rise of *Pdv*, in a small circumscribed area, may cause the differentiation of the lentoid.

It must be admitted that such a presentation neglects the normal elaboration of the organ rudiments. If we distinguish two phases in the development of an organ-rudiment from the germ layer, *viz.*, the phase of determination of the basic pattern of the organ, and the phase of elaboration of this pattern, the above discussion deals only with the former. For the latter we must perhaps take other factors into account, which need not be specific. In the isolate from earlier developmental stages the phase of elaboration seems to be absent, and the structures developed within it are, in no case, normally elaborated. As this imperfection of elaboration occurs also in the isolate with the living organizer, we cannot ascribe it wholly to an inadequate nature of the primary inductor.

What, therefore, will be expected from the theory when the same treatment is applied to the ventral marginal zone of the early gastrula? This germ region can be characterized as having a low *Pdv* and an intermediate *Pcc*, or more precisely, as being endowed with the inherent capacity to raise its *Pcc* to an intermediate level, because it gives rise to the ventral differentiations of the trunk region in normal development and also in isolation (control series II). The ammonia-treatment must, in this case, change only *Pdv* leaving the *Pcc* unaffected. The results may be the maximal value of *Pdv* and an intermediate value of *Pcc*. This means the

[4] It may be added in this connection that the theory was proposed at the time when the works of Holtfreter (1944, 1945 and 1947) were unknown in Japan.

differentiation of the axial organs of the trunk. Our results (experimental series) fulfill the theoretical expectation. The regional differences of *Pdv* within the isolated mesoderm may account for the occurrence of different tissues side by side; the maximal value corresponding to the notochord, the next highest value to the muscle and the intermediate one to the pronephros. That a good differentiation of the myofibrils occurs only in the presence of a notochord may be interpreted by the assumption that this differentiation presupposes a gradual rise of *Pdv* in the later stages, caused by the notochord which plays the role of dorso-ventral mediator owing to its special histogenesis. But we must not neglect the possibility that stretching of the muscle, which is caused by the notochord, plays an important role in the formation of its fibrils.

It is well known that many physiological and histochemical reactions show clear dorso-ventral gradients which are superimposable upon each other. This type of gradient is, for instance, exhibited by cytochrome oxidase, sulfhydryl groups of protein, ribonucleic acid, alkaline phosphatase and acetalphosphatide. Recent studies make it probable that at least some of these reactions are bound to submicroscopic granules called microsomes (Brachet, 1949). Studying the effect of cytolysis on the behavior of cytoplasmic ribonucleic acid, J. Brachet (1946) finds that the basophilia of the cytoplasm of embryonic cells of amphibia markedly increase when exposed to an abnormal salt solution, and that this basophilic material can be digested away by a dilute solution of crystalline ribonuclease. Holtfreter (1948) reports that an exposure of isolated embryonic tissue to salt solution of high pH produces "appearance and precipitation of basophilic granules in the previously hyaline portion of the protoplasm." From these observations it seems probable that ammonia-treatment causes an increase of the basophilic granules in the ectoderm as well as in the mesoderm. If this is the case, then there exists a clear parallel between the concentration of basophilic granules and *Pdv* in experimental as well as in normal conditions. In both cases the dorsal mode of development occurs in the region with high granular content, and the ventral mode in the region with low content. This may mean that *Pdv* is nothing but the capacity of cells to maintain or produce a certain concentration of these granules. Recent data on the role of ribonucleic acid in many cytological elaborations and formation of fiber protein (Brachet, 1949), make it probable that differentiations of neurofibrils, myofibrils and notochord vacuoles (which occur exclusively in the region of high *Pdv*) are in some way connected with the basophilic granules.

Summary

(1) Control series I. The ectoderm of the early gastrula of *Triturus pyrrhogaster* was isolated and exposed for a short time to a solution of ammonia and then cultured in Holtfreter solution. The isolate differentiated structures which can be characterized as cephalic ectodermal rudiments including brain, eye, nose and frontal glands. Otic vesicles, spinal cord and mesodermal structures were wholly absent.

(2) Control series II. The ventral marginal zone of the early gastrula was isolated and cultured in Holtfreter solution. It differentiated blood island, blood vessels and mesothelium and nephric tubules.

(3) Experimental series. The isolated ventral marginal zone was treated with ammonia solution and cultured in Holtfreter solution. It gave rise to notochord

and muscle as well as nephric tubules. The development of the blood island, mesothelium and blood vessels was almost completely suppressed.

(4) Results were interpreted from the standpoint of the double-potential theory (Yamada, 1947).

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